


RESEARCH ARTICLE

Pre-clinical evaluation of dual targeting of the GPCRs CaSR and V2R as therapeutic strategy for autosomal dominant polycystic kidney disease

Annarita Di Mise¹  | Xiaofang Wang² | Hong Ye² | Lorenzo Pellegrini³ | Vicente E. Torres² | Giovanna Valenti¹

¹Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari Aldo Moro, Bari, Italy

²Division of Nephrology and Hypertension, Mayo Clinic, Rochester, Minnesota, USA

³Palladio Biosciences, Inc., Horsham, Pennsylvania, USA

Correspondence

Annarita Di Mise and Giovanna Valenti, Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari Aldo Moro, via Orabona, 4, 70125, Bari, Italy.
 Email: annarita.dimise@uniba.it (A. D. M.); giovanna.valenti@uniba.it (G. V.)

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD), caused by mutations of *PKD1* or *PKD2* genes, is characterized by development and growth of cysts causing progressive kidney enlargement. Reduced resting cytosolic calcium and increased cAMP levels associated with the tonic action of vasopressin are two central biochemical defects in ADPKD. Here we show that co-targeting two GPCRs, the vasopressin V2 receptor (V2R) and the calcium sensing receptor, using the novel V2R antagonist lixivaptan in combination with the calcimimetic R-568, reduced cyst progression in two animal models of human PKD. Lixivaptan is expected to have a safer liver profile compared to tolvaptan, the only drug approved to delay PKD progression, based on computational model results and initial clinical evidence. PCK rat and *Pkd1*^{RC/RC} mouse littermates were fed without or with lixivaptan (0.5%) and R-568 (0.025% for rats and 0.04% for mice), alone or in combination, for 7 (rats) or 13 (mice) weeks. In PCK rats, the combined treatment strongly decreased kidney weight, cyst and fibrosis volumes by 20%, 49%, and 73%, respectively, compared to untreated animals. In *Pkd1*^{RC/RC} mice, the same parameters were reduced by 20%, 56%, and 69%, respectively. In both cases the combined treatment appeared nominally more effective than the individual drugs used alone. These data point to an intriguing new application for two existing drugs in PKD treatment. The potential for synergy between these two compounds suggested in these animal studies, if confirmed in appropriate clinical investigations, would represent a welcome advancement in the treatment of ADPKD.

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; AMPK, AMP-activated protein kinase; ARPKD, autosomal recessive polycystic kidney disease; CaSR, calcium sensing receptor; CFTR, cystic fibrosis transmembrane conductance regulator; ciPTEC, conditionally immortalized proximal tubular epithelial cells; CKD, chronic kidney disease; FDA, Food and Drug Administration; GPCRs, G protein coupled receptors; LXV, lixivaptan; mTOR, mechanistic target of rapamycin; PC1, polycystin-1; PC2, polycystin-2; PTH, parathyroid hormone; REMS, risk evaluation and mitigation strategy; V2R, vasopressin V2 receptor.

Vicente E. Torres and Giovanna Valenti contributed equally to this work.

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KEYWORDS

calcimimetics, calcium-sensing receptor, GPCRs, polycystic kidney disease, vaptans, vasopressin V2 receptor

1 | INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a dominant inherited disease characterized by progressive and bilateral cyst development and growth in the kidneys, but also in the liver and other organs.¹ By the age of 60 years, most patients develop kidney failure and 50% of them require renal replacement therapy.² ADPKD is caused primarily by loss-of-function mutations in the *PKD1* or *PKD2* genes, encoding polycystin-1 (PC1) and polycystin-2 (PC2), respectively. Loss-of-function of PC1 or PC2 is associated with decreased cytosolic calcium content and increased intracellular cAMP levels, with consequent disruption of molecular pathways controlling tubulogenesis, cellular proliferation, and fluid secretion, eventually causing the development of fluid-filled cysts.^{1,3}

Currently, the only drug that is specifically approved for the treatment of patients with rapidly progressive ADPKD in Europe, Japan, Canada and the United States is tolvaptan, a vasopressin V2 receptor (V2R) antagonist. Two phase 3 clinical studies in ADPKD patients demonstrated its efficacy in reducing total kidney volume growth and slowing eGFR decline.^{4,5} However, tolvaptan was also found to cause liver toxicity in ADPKD patients.⁶ On this basis, the US Food and Drug Administration recommended using tolvaptan only under a risk evaluation and mitigation strategy, which is aimed at preventing serious and potentially life-threatening liver injury.^{7,8} Therefore, although tolvaptan is effective in slowing ADPKD progression in patients, is important to develop interventions that are both effective and safe especially in lifelong treatments.

Recently, a novel V2R antagonist, lixivaptan, which had been initially developed for the treatment of hyponatremia,⁹ was shown to reduce kidney cystic burden and cAMP levels in PCK rats, an orthologous model of human PKD.¹⁰ In cortical collecting duct cells, lixivaptan prevented the increase in intracellular cAMP, aquaporin-2 trafficking and water reabsorption in response to vasopressin, providing a mechanistic explanation for the aquaretic effect observed in vivo.¹¹

In addition, lixivaptan and its three major metabolites were represented in DILIsym, a quantitative systems toxicology platform model of drug-induced liver injury, in order to predict lixivaptan's potential risk of hepatotoxicity at doses intended for the treatment of ADPKD.¹² The results of the DILIsym simulation for lixivaptan, compared

to the previously published simulation for tolvaptan,¹³ suggest that lixivaptan is less likely to induce hepatotoxicity than tolvaptan at their respective therapeutic doses for ADPKD treatment.¹² Subsequently, lixivaptan was tested in a phase 2 clinical trial in ADPKD patients with chronic kidney disease (NCT03487913). Preliminary results of the study confirmed that lixivaptan is a potent V2R antagonist in ADPKD patients. The study also defined the dose range for an open-label clinical trial designed to assess the liver safety of lixivaptan in subjects who previously experienced liver chemistry test abnormalities while treated with tolvaptan (NCT04152837), and for an upcoming Phase 3, double-blind, placebo-controlled, randomized trial to demonstrate the efficacy and safety of lixivaptan in subjects with ADPKD (NCT04064346).

Two of the most crucial molecular dysregulations in ADPKD which are considered pro-proliferative events, i.e., decreased cytosolic calcium content and increased intracellular cAMP levels,¹⁴ are both modulated in the kidney by the activation of the extracellular calcium-sensing receptor (CaSR).¹⁵ CaSR is a G-protein-coupled receptor which senses changes in extracellular calcium concentrations and regulates parathyroid hormone secretion and renal tubular calcium reabsorption to maintain serum calcium levels within the normal range.¹⁵ Recently, we have shown that conditionally immortalized human proximal tubular epithelial cells (ciPTEC) isolated from urine of a healthy subject or of a *PKD1* patient, or upon stable down-regulation of *Pkd1*, express a functional CaSR.^{16,17} Interestingly, specific activation of CaSR elicited by its allosteric modulator, the calcimimetic R568, induced an increase of cytosolic Ca^{2+} , a decrease in intracellular cAMP level, and a reduction of mechanistic target of rapamycin activity.¹⁷ In addition, R568 rescued the cell energy status by increasing intracellular ATP levels and mitochondrial calcium content.¹⁸ In animal models of PKD (Cy/+ Han:SPRD rats and pcy mice), calcimimetics inhibited late-stage cyst growth by increasing intracellular calcium.^{19,20} Conversely, in other animal models (PCK rats and *Pkd2*^{WS25/-} mice), no significant effect of calcimimetics was reported—except for reduced fibrosis—possibly because the potential positive effect of the calcimimetics was offset by marked hypocalcemia.²¹ It is currently unknown whether reduced doses of calcimimetic could retain efficacy in the treatment of ADPKD without eliciting the potential detrimental effect of hypocalcemia.

In the present study, we investigated the effect of combination treatment with the calcimimetic R568 and lixivaptan in two animal models of human PKD, PCK rats and *Pkd1*^{RC/RC} mice, using a lower dose of R568, to examine the relative role of their two GPCR targets. Specifically, we evaluated the endpoint manifestations of disease such as kidney weight, kidney cysts, kidney fibrosis volume and renal cAMP.

2 | MATERIALS AND METHODS

2.1 | Experimental animals and study design

All experiments were performed in PCK rats and *Pkd1*^{RC/RC} mice maintained in the animal facilities of the Department of Veterinary Medicine of the Mayo Clinic (Rochester, MN, USA). All animal procedures were approved by the Institutional Animal Care and Utilization Committee.

The PCK rat, a Sprague-Dawley strain, is a model orthologous to ARPKD. It is characterized by an aberrant structure and function of the fibrocystin protein, which is caused by a splicing mutation that leads to a frameshift in *Pkhd1* gene, and which in turn results in progressive PKD.^{22,23} The *Pkd1*^{RC/RC} mouse model, inbred into the C57BL/6 background, is homozygous for the hypomorphic *Pkd1* p.R3277C allele, orthologous to ADPKD and exhibits slowly progressive PKD.²⁴

The animals were fed ground rodent chow ad libitum (Labdiet 5053, Purina Mills, Richmond, IN, USA). At the 4th week of age, they were divided into four groups on a control diet or a diet containing lixivaptan and/or R568. Rats ($n = 80$, 10 animals per group and gender) received ground rodent chow containing 0.5% lixivaptan, 0.025% R568, 0.5% lixivaptan and 0.025% R568 together, or rodent chow without drugs (control group) for 7 weeks. Mice ($n = 80$, 10 animals per treatment group and gender) were fed with ground rodent chow containing 0.5% lixivaptan, 0.04% R568, 0.5% lixivaptan and 0.04% R568 together, or rodent chow without drugs (control group) for 13 weeks. One week before the scheduled sacrifice, animals were housed in metabolic cages to collect 24-h urine outputs. At 10 weeks (rats) or 16 weeks (mice) of age, animals were weighed and anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. Blood was obtained by cardiac puncture and was used for plasma calcium, creatinine and urea levels determination. The right kidney was placed into pre-weighed vials containing 10% formaldehyde/phosphate buffer saline (pH 7.4). Tissues were embedded in paraffin for histological and histomorphometric analysis. The left kidneys were

immediately frozen in liquid nitrogen for cAMP or PKA activity measurements.

2.2 | cAMP content and PKA activity of whole kidneys

For cAMP analysis, whole kidneys were ground to fine powder under liquid nitrogen and homogenized in 0.1 M HCl at room temperature. Total protein content was determined with the BCA Protein Assay Kit (Pierce, IL, USA). Samples were centrifuged at 10 000 rpm for 10 min and the supernatant was used to measure cAMP levels with Direct cAMP ELISA kit, according to the manufacturer's instructions (Enzo Life Sciences, Inc, Farmingdale, NY, USA).

PKA activity determination was assessed by PKA Colorimetric Activity Kit (ThermoFisher Scientific Inc, Monza, Italy). Whole kidney slices were homogenized in Lysis Buffer supplied by the manufacturer. After total protein concentration measurement, samples were centrifuged at 10 000 rpm for 10 min at 4°C and the supernatant was used for PKA activity analysis, performed following manufacturer's instructions.

2.3 | Gel electrophoresis and immunoblotting

Proteins were separated on 10% polyacrylamide gels (Bio-Rad Laboratories, Inc, Hercules, CA, USA) under reducing conditions. Protein bands were electrophoretically transferred onto Immobilon-P membranes (Merck Millipore, Darmstadt, Germany) and incubated with primary antibodies overnight. Anti-Phospho-AMPK (Thr172 of its α subunit) and anti-Tot AMPK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-ERK 1/2 and anti-Phospho-ERK 1/2 (Thr185/Tyr187) were from Merck Millipore. Bands were normalized to total protein using stain-free technology. Densitometry analysis was performed using ImageLab (Bio-Rad Laboratories, Inc, Hercules, CA, USA) and analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

2.4 | Histomorphometric and histological analysis

Tissue cross-sections (5 μ m) of whole kidney were stained with hematoxylin-eosin, for cyst volume measurement, and picosirius red for collagen, to analyze fibrosis.²⁵ Stained sections were visualized under a Nikon

microscope and digital images were acquired using a high-resolution color digital camera (Nikon DS-Ri1). Mice cyst and fibrosis volumes were calculated using three cross-sections per kidney. For renal fibrosis, sequential images were collected using a 10× objective for rats and a 20× objective for mice sections, moving from one end of the available cortex to the other, without overlapping, excluding renal medulla. Analysis of light microscopy images was performed using the NIS-Elements AR software system (Nikon, Elgin, IL, USA), applying a colored threshold to separate the objects from their background. Renal cyst volume was calculated from the percentage cystic area of the sections adjusted to kidney weight. Fibrosis volume was calculated by measuring the percentage fibrotic area of 8 cortical images adjusted to kidney weight.

2.5 | Calcium and phosphorus determinations

Plasma calcium and phosphorus were measured using a Hitachi Modular System.

2.6 | Statistical analysis

One-way ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis. All values

are expressed as means \pm SEM. A difference of $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Effect of combined lixivaptan and R568 treatment on cAMP content in whole kidneys

Increased renal cAMP levels are one of the principal biochemical hallmarks of ADPKD. In PCK rats, both lixivaptan or R568 monotherapy displayed a trend in reducing total renal cAMP (33.3% reduction vs. control in lixivaptan treated animals and 20.5% reduction vs. control in R568 treated animals); however, only the combined treatment induced a statistically significant reduction in cAMP levels (38.7%, $p = .033$) compared to animals fed with standard diet (Figure 1A).

In *Pkd1*^{RC/RC} mice, renal cAMP levels were indirectly determined by measuring PKA activity (Figure 1B). Once again, whereas both lixivaptan and R568 monotherapy showed a trend toward reduction of PKA activity, only the combined lixivaptan and R568 treatment statistically significantly reduced PKA activity (65.3% vs. CTR, $p = .024$; Figure 1B). Interestingly, in this animal model of PKD, R568 appeared to have a more potent effect (51.7% reduction) than lixivaptan (29.5% reduction) relative to control animals, although the difference did not reach statistical significance.

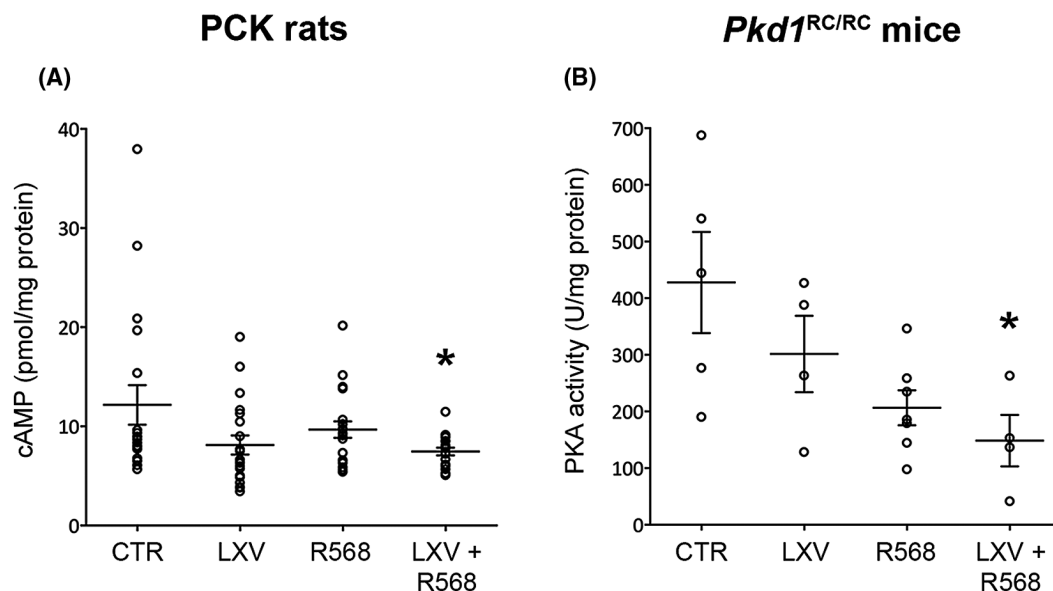


FIGURE 1 Effect of lixivaptan and R568 treatment on renal cAMP levels or PKA activity. (A) Renal cAMP in PCK rats, expressed as pmol cAMP on mg of total proteins. (B) PKA activity in kidney homogenates in *Pkd1*^{RC/RC} mice, measured as units (U) on mg of total proteins. Data are expressed as means \pm SEM, * $p < .05$ versus CTR

3.2 | Effect of combined lixivaptan and R568 treatment on AMPK and ERK1/2 expression

AMP-activated protein kinase (AMPK) is a key regulator of the defective aerobic glycolysis and proliferative processes in PKD.²⁶ Reduced AMPK signaling has been observed in PKD and, consistent with this observation, AMPK activation has been proved to significantly reduce cystic growth in both in vitro and ex vivo models of renal cystogenesis.^{27,28} The effect of the combined treatment on active AMPK was evaluated by measuring the levels of AMPK phosphorylated at residue Thr172 of its α subunit. Interestingly, in PCK rats, AMPK activity was significantly increased under combined treatment (CTR = 1.0 ± 0.1 , LXV + R568 = 1.7 ± 0.2 , $p < .01$ vs. CTR; LXV =

1.1 ± 0.1 , R568 = 1.3 ± 0.1 , n.s. vs. CTR; Figure 2A,B). In *Pkd1^{RC/RC}* mice, the two drugs in combination induced a five-fold increase in pAMPK levels (CTR = 1.0 ± 0.1 , LXV + R568 = 5.1 ± 0.7 , $p < .0001$; Figure 2C,D). Both lixivaptan and R568 monotherapy increased pAMPK, although only treatment with calcimimetic resulted in a statistically significant increase compared to CTR (LXV = 2.2 ± 0.3 , n.s. vs. CTR; R568 = 3.3 ± 0.4 , $p < .01$ vs. CTR; Figure 2D). Combined treatment with the two drugs had a clear significant effect in increasing pAMPK expression with respect to both lixivaptan and R568 monotherapy ($p < .001$ vs. LXV, $p < .05$ vs. R568).

Various pro-proliferative pathways are upregulated in ADPKD. Several studies demonstrated the activation of the MAPK/ERK pathway in cellular and animal PKD models.^{29,30} Evaluation of pERK1/2 levels revealed that

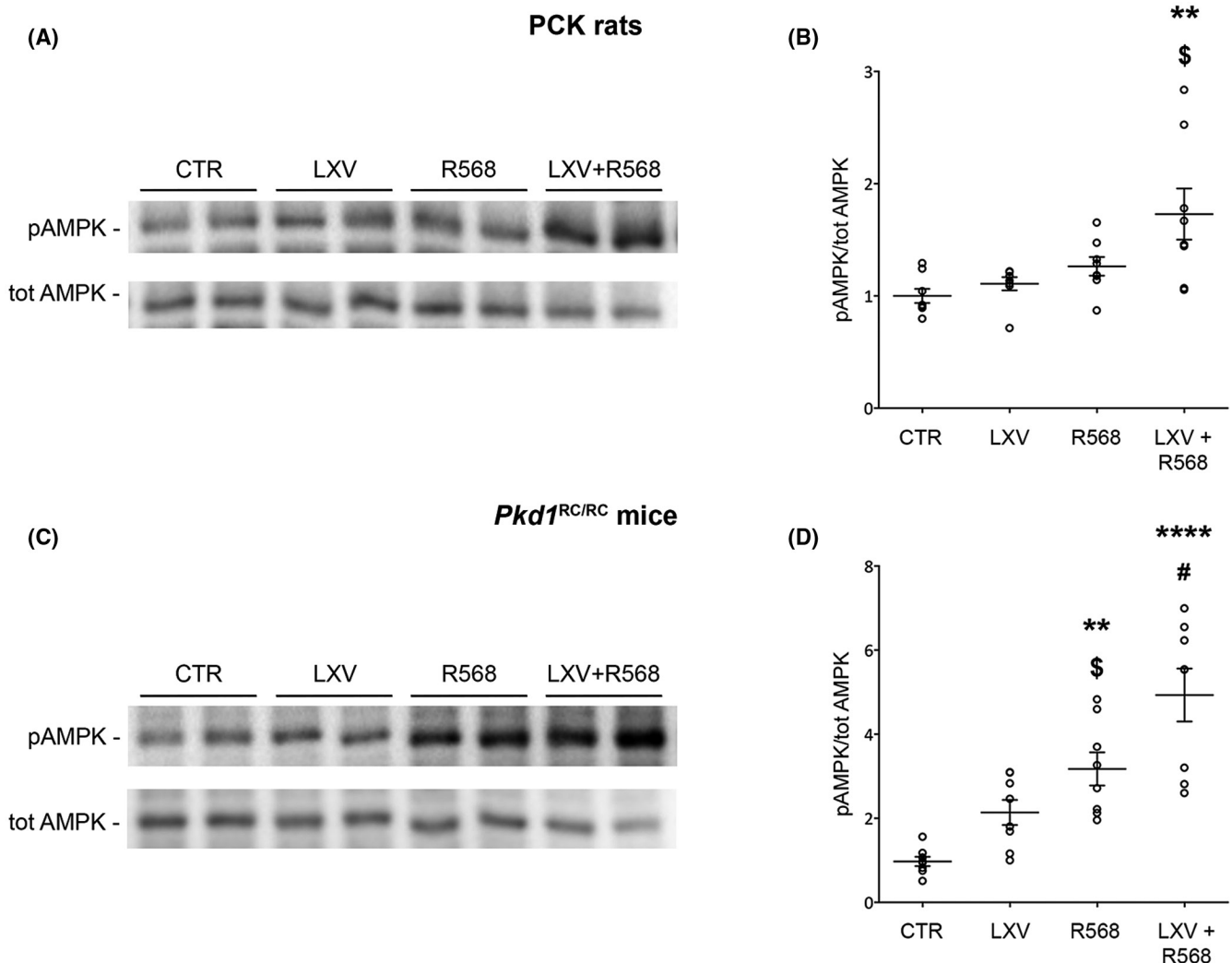


FIGURE 2 AMPK phosphorylation levels in PCK rats and *Pkd1^{RC/RC}* mice. (A–C) Representative blots for total AMPK or for pT172-AMPK (pAMPK). (B) Densitometric analysis and statistical studies (means \pm SEM) in PCK rats ($n = 8$, 4 animals per group and gender), ** $p < .01$ versus CTR, $^{\$}p < .05$ versus LXV. (D) Densitometric analysis and statistical studies (means \pm SEM) in *Pkd1^{RC/RC}* mice ($n = 8$, 4 animals per group and gender), **** $p < .0001$ versus CTR, $^{\#}p < .001$ versus LXV, ** $p < .01$ versus CTR, $^{\$}p < .05$ versus LXV + R568

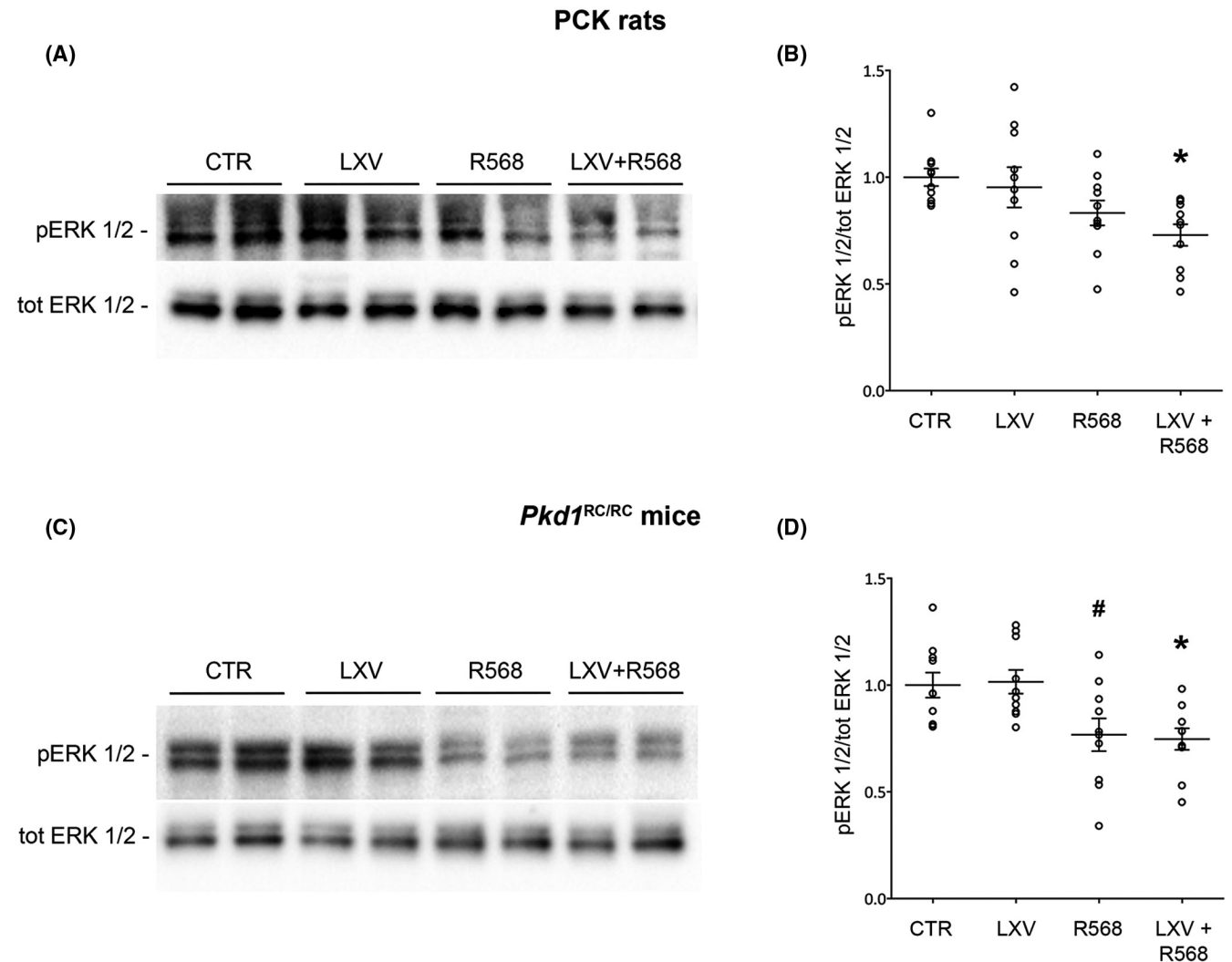


FIGURE 3 ERK phosphorylation levels in PCK rats and *Pkd1^{RC/RC}* mice. (A–C) Representative blots for total ERK1/2 or ERK1/2 phosphorylated at Thr185/Tyr187 (pERK 1/2). (B) Densitometric analysis and statistical studies (means \pm SEM) in PCK rats ($n = 10$, 5 animals per group and gender), * $p < .01$ versus CTR. (D) Densitometric analysis and statistical studies (means \pm SEM) in *Pkd1^{RC/RC}* mice ($n = 10$, 5 animals per group and gender), * $p < .01$ versus CTR and LXV, # $p < .01$ versus LXV

cotreatment with lixivaptan and R568 in both animal models resulted in a significant reduction in pERK1/2 levels (PCK rats: CTR = 1.0 ± 0.04 , LXV + R568 = 0.7 ± 0.05 , $p < .01$ vs. CTR; LXV = 0.95 ± 0.09 , R568 = 0.8 ± 0.05 , n.s. vs. CTR, **Figure 3A,B**; *Pkd1^{RC/RC}* mice: CTR = 1.0 ± 0.06 , LXV + R568 = 0.7 ± 0.05 , $p < .01$ vs. CTR and LXV; LXV = 1.02 ± 0.05 n.s. vs. CTR, R568 = 0.8 ± 0.08 n.s. vs. CTR, $p < .01$ vs. LXV, **Figure 3C,D**).

3.3 | Effect of combined lixivaptan and R568 treatment on renal cyst burden and fibrosis

The effect of the treatments on the ADPKD phenotype was evaluated by analyzing kidney weight (expressed as percentage of body weight), cyst volume and fibrosis

volume. In both animal models, untreated animals displayed a typical PKD phenotype characterized by enlarged kidneys and relevant renal cysts development and fibrosis, as expected.

In PCK rats, treatment with either lixivaptan or R568 statistically significantly reduced kidney weight (normalized to body weight, **Figure 4A**). Interestingly, the reduction in kidney weight in rats treated with lixivaptan combined with R568 versus untreated animals reached a higher level of statistical significance compared to the monotherapy groups (LXV 19.7% reduction, $p = .0000074$ vs. CTR; R568 9.2% reduction, $p < .05$ vs. CTR; combined treatment 20.4% reduction, $p = .0000047$ vs. CTR) (**Figure 4A**).

Figure 4B shows representative tissue cross-sections of whole kidney from the four experimental conditions after staining with hematoxylin-eosin. As expected, kidney

PCK rats

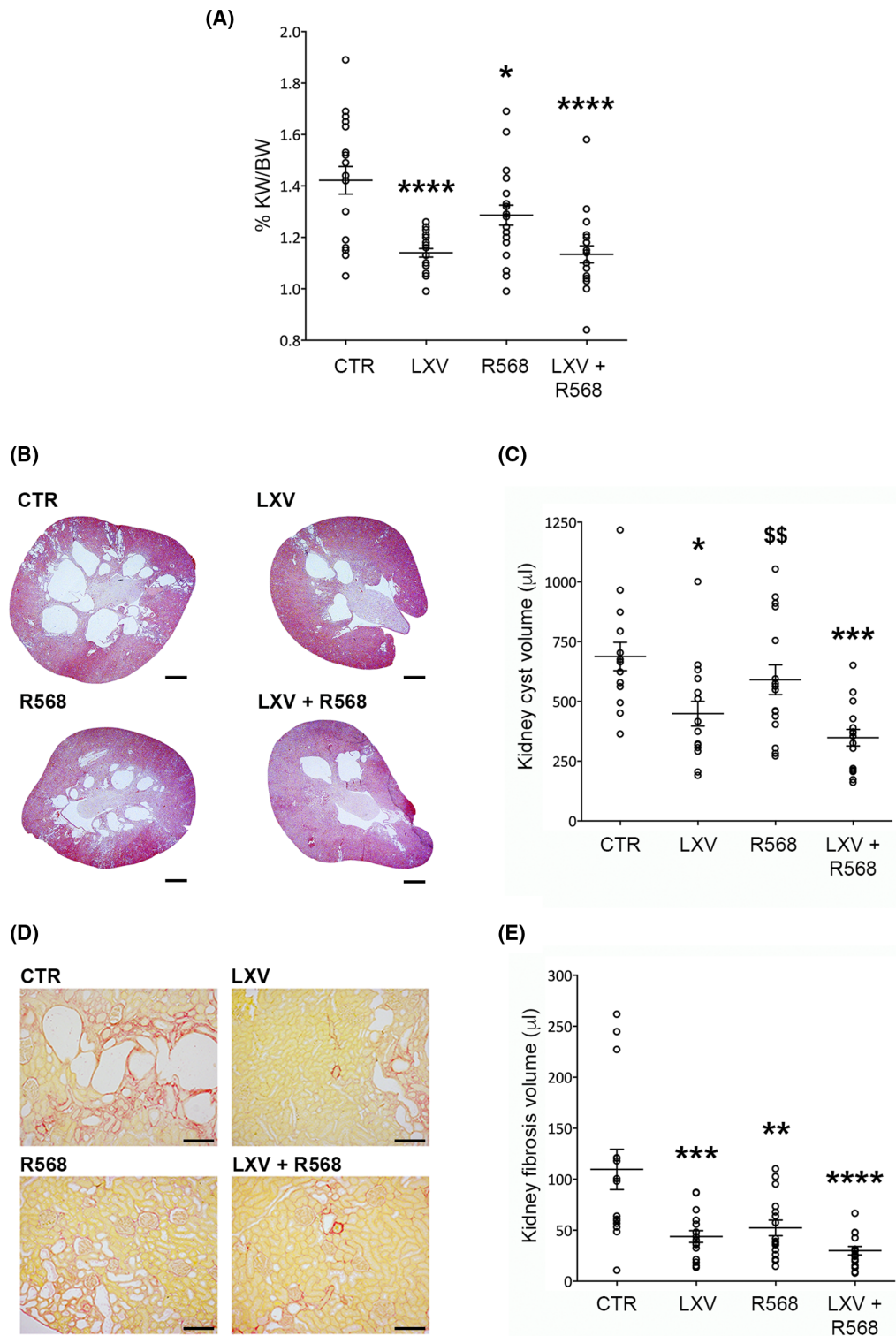


FIGURE 4 Effect of lixivaptan and R568 treatment on renal morphology in PCK rats. (A) Evaluation of the effect of the drugs on kidney weight expressed as percentage of body weight. Data are expressed as means \pm SEM, **** p < .0001 versus CTR, * p < .05 versus CTR or LXV or LXV + R568. (B) Representative histological images of kidney cross-sections (scale bars: 2 mm) stained with hematoxylin-eosin for cyst volume analysis, showed in the histogram (C). Data are expressed as means \pm SEM, * p < .05, *** p < .001 versus CTR, \$\$ p < .01 versus LXV + R568. (D) Representative histological images of transverse kidney sections (scale bars: 200 μ m) stained with picrosirius red for fibrosis measurement, quantified in the histogram (E). Data are expressed as means \pm SEM, *** p < .001, ** p < .01, **** p < .0001 versus CTR

sections from control animals exhibit the presence of large cysts mainly restricted to the medullary region. Cyst burden appeared qualitatively reduced in all treated groups. Quantitative analysis of kidney cyst volume (Figure 4C) revealed that lixivaptan alone was more effective (34.8% reduction, $p < .05$ vs. CTR) compared to R568 (14.1% reduction, n.s. vs. CTR) in reducing cyst burden. Of note, PCK rats treated with lixivaptan and R568 together showed the highest degree of cyst volume reduction (49.4% reduction, $p < .001$ vs. CTR).

Besides cyst burden, a major pathological feature of PKD is the development of interstitial inflammation and fibrosis, which is associated with accumulation of inflammatory cells.^{31–33} Interestingly, qualitative (Figure 4D) and quantitative (Figure 4E) evaluation of kidney fibrosis by picrosirius red staining for collagen demonstrated that both lixivaptan and R568 monotherapy had a strong, statistically significant effect in reducing renal fibrosis volume (LXV 60% reduction, $p < .001$ vs. CTR; R568 52.3% reduction, $p < .01$ vs. CTR) (Figure 4E). Of interest, the combined treatment had the strongest effect in reducing cyst fibrosis (72.7%, $p < .0001$ vs. CTR) (Figure 4E).

The effect of the combined lixivaptan and R568 treatment on renal cysts was also tested in *Pkd1*^{RC/RC} mice, a PKD1 model characterized by gradually increasing cyst burden, which makes it optimal for therapeutic testing.²⁴ In *Pkd1*^{RC/RC} mice, only lixivaptan alone or in combination with R568 significantly reduced kidney weight; however, the combined treatment induced a nominally higher reduction, and the reduction reached a higher level of statistical significance, compared to animals treated with lixivaptan alone (Figure 5A). Specifically, kidney weight decreased by 15.5% in animals treated with lixivaptan alone ($p < .01$ vs. CTR), and by 20.1% in animals fed with lixivaptan and R568 diet ($p < .0001$ vs. CTR). R568 alone induced a non-significant decrease (8.1%) compared to control animals. Histochemical qualitative (Figure 5B) and quantitative (Figure 5C) analysis of kidney cyst volume from tissue cross-sections of whole kidneys demonstrated that both lixivaptan and R568 monotherapy were effective in reducing kidney cyst burden in *Pkd1*^{RC/RC} mice; however, in animals treated with the two drugs in combination, once again a nominally stronger effect was obtained and the comparison with control animals reached a higher level of statistical significance (LXV 38.9%, $p < .05$ vs. CTR; R568 42.7%, $p < .01$ vs. CTR; combined treatment 55.5%, $p < .001$ vs. CTR, Figure 5C). An analogous result was observed upon analysis of renal fibrosis volume after picrosirius red staining (Figure 5D). Lixivaptan and R568 monotherapy had a similar significantly strong effect in reducing renal fibrosis volume compared to animals fed with regular diet (LXV 64.4% reduction, $p < .001$ vs. CTR; R568 64.9% reduction, $p < .001$ vs. CTR). Importantly,

once again the combined treatment showed a nominally greater effect, and the comparison with control animals reached a higher level of statistical significance, in reducing renal fibrosis (69.1% reduction, $p < .0001$ vs. CTR, Figure 5E).

Taken together, these data, summarized in Tables 1 and 2, demonstrate that combination treatment with lixivaptan and R568 has similar effects in two different PKD animal models and may be more effective in reducing disease manifestations than the single drugs used as monotherapies.

To evaluate whether the combined treatment had different effects in males and females, the data were also analyzed considering the sex. For most outcomes (cAMP, KW/BW, cyst and fibrosis volumes), the significance is randomly retained in males or females. In the cases where the significance is lost, due to the small sample size, there is a clear trend in the same direction and the effects of treatment are confirmed (Figures S1–S3).

3.4 | Effect of combined lixivaptan and R568 treatment on parameters related to renal function

Tables 1 and 2 report the effect of combined lixivaptan and R568 treatment on parameters related to renal function in PCK rats and *Pkd1*^{RC/RC} mice, respectively. No effect on total body weight was observed in any of the treatment groups compared to controls (untreated PKD animal models).

In both PKD models, PCK rats and *Pkd1*^{RC/RC} mice, lixivaptan treatment caused a 3-fold increase in 24-h urine output compared to untreated control animals, confirming the strong aquaretic effect of the drug.¹⁰ Conversely, R568 alone used at 0.025% in rats and 0.04% in mice had no effect on the 24-h diuresis both in PCK rats and *Pkd1*^{RC/RC} mice. This result is not in agreement with what was observed in a previous study in PCK rats treated with R568 0.05% or 0.1%²¹ and is likely due to the reduced dose employed in the present study. Finally, in both animal models, combination therapy with lixivaptan and R568 did not increase urine output compared to lixivaptan monotherapy, confirming that the low doses of calcimimetic in this study do not exacerbate the aquaretic effect of lixivaptan.

In both animal models, there was no effect on plasma urea and creatinine with any treatment, either alone or combined, although a trend toward a decrease in plasma creatinine in PCK rats and in plasma urea in *Pkd1*^{RC/RC} mice was observed with the combined treatment with the two drugs (data not shown). Plasma phosphorus was significantly increased in PCK rats, but not in *Pkd1*^{RC/RC} mice, with R568 treatment either alone or combined

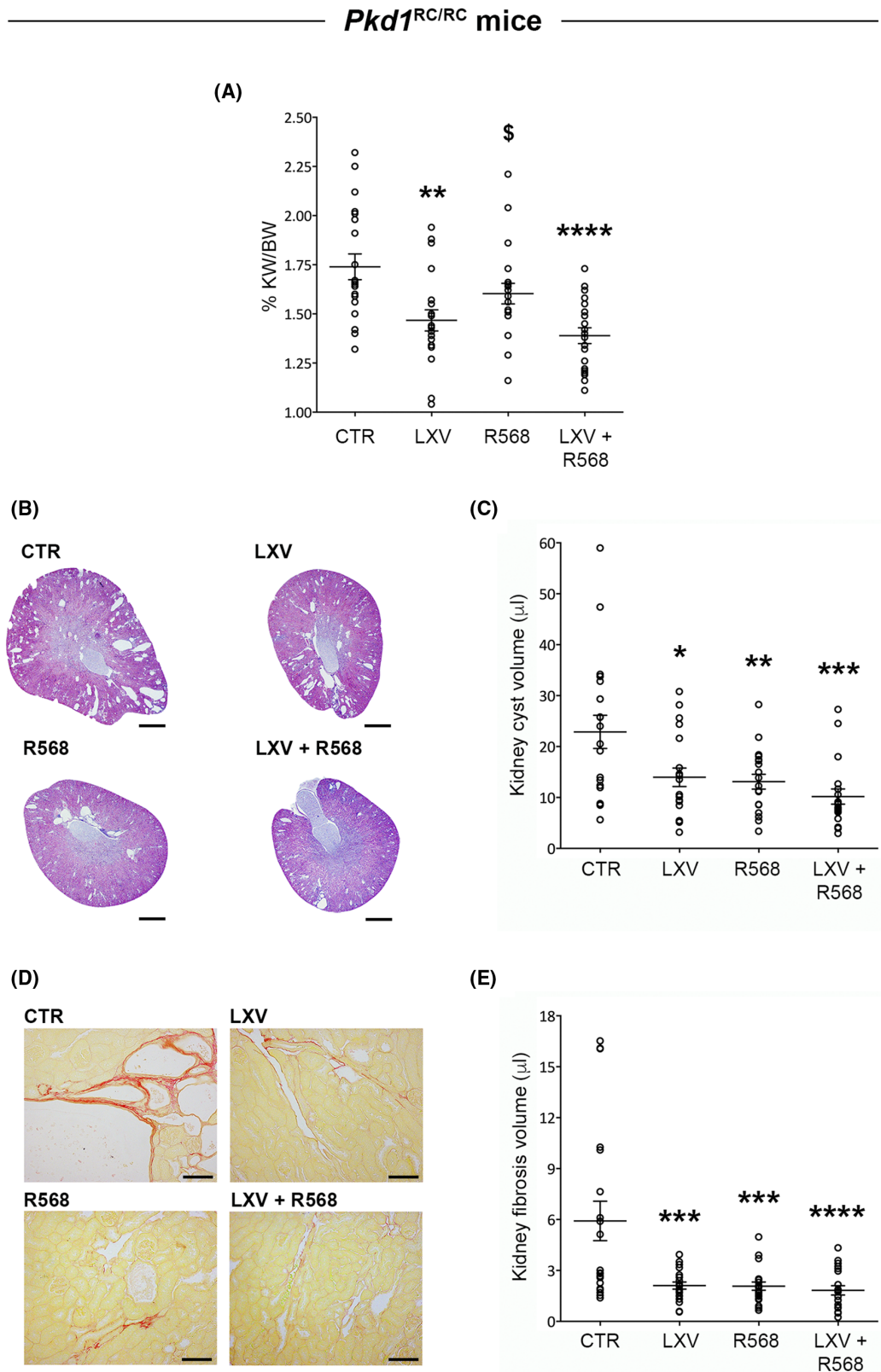


FIGURE 5 Lixivaptan and R568 treatment effect on renal morphology in *Pkd1*^{RC/RC} mice. (A) Evaluation of the effect of the drugs on kidney weight expressed as percentage of body weight. Data are expressed as means \pm SEM, ** $p < .01$, **** $p < .0001$ versus CTR, \$ $p < .05$ versus LXV + R568. (B) Representative histological images of transverse kidney sections (scale bars: 1 mm) stained with hematoxylin-eosin for cyst volume analysis, showed in the histogram (C). Data are expressed as means \pm SEM, * $p < .05$, ** $p < .01$, *** $p < .001$ versus CTR. (D) Representative histological images of kidney cross-sections (scale bars: 100 mm) stained with picrosirius red for fibrosis measurement, quantified in the histogram (E). Data are expressed as means \pm SEM, *** $p < .001$, **** $p < .0001$ versus CTR

TABLE 1 Effect of lixivaptan and R568 on parameters involved in PKD development in PCK rats

Parameters	Values \pm SEM	p values			
		CTR (n = 20)	LXV (n = 20)	R568 (n = 20)	LXV + R568 (n = 20)
Kidney weight/BW (%)					
CTR	1.42 \pm 0.05	–	<.0001*	.0627	<.0001*
LXV	1.14 \pm 0.02	<.0001*	–	.0345*	.9995
R568	1.29 \pm 0.04	.0627	.0345*	–	.0256*
LXV + R568	1.13 \pm 0.03	<.0001*	.9995	.0256*	–
cAMP (pmol/mg protein)					
CTR	12.17 \pm 1.99	–	.0819	.4498	.0341*
LXV	8.12 \pm 0.96	.0819	–	.7807	.9792
R568	9.68 \pm 0.83	.4498	.7807	–	.5493
LXV + R568	7.46 \pm 0.38	.0341*	.9792	.5493	–
Cystic volume (μ l)					
CTR	687.9 \pm 59.08	–	.0134*	.5787	.0002*
LXV	448.8 \pm 51.83	.0134*	–	.2249	.5229
R568	590.7 \pm 62.27	.5787	.2249	–	.0086*
LXV + R568	348.3 \pm 34.41	.0002*	.5229	.0086*	–
Fibrotic volume (μ l)					
CTR	109.6 \pm 19.79	–	.0004*	.0024*	<.0001*
LXV	43.85 \pm 5.78	.0004*	–	.9442	.795
R568	52.29 \pm 7.62	.0024*	.9442	–	.4584
LXV + R568	29.93 \pm 4.14	<.0001*	.795	.4584	–
Plasma phosphorus (mg/dl)					
CTR	8.61 \pm 0.31	–	.9854	.0295*	.0048*
LXV	8.45 \pm 0.34	.9854	–	.0103*	.0013*
R568	9.96 \pm 0.35	.0295*	.0103*	–	.9464
LXV + R568	10.22 \pm 0.31	.0048*	.0013*	.9464	–
24 h urine volume (ml)					
CTR	14.72 \pm 0.7	–	<.0001*	.3414	<.0001*
LXV	48.37 \pm 2.56	<.0001*	–	<.0001*	.4656
R568	10.65 \pm 0.72	.3414	<.0001*	–	<.0001*
LXV + R568	44.88 \pm 1.97	<.0001*	.4656	<.0001*	–
Body weight (g)					
CTR	325.3 \pm 19.41	–	.9499	.9997	.8991
LXV	311.3 \pm 19.37	.9499	–	.9703	.9985
R568	322.8 \pm 17.67	.9997	.9703	–	.9301
LXV + R568	307.2 \pm 16.63	.8991	.9985	.9301	–

Note: Data are expressed as means \pm SEM.

* $p < .05$.

with lixivaptan. Importantly, plasma calcium measured in *Pkd1*^{RC/RC} mice was not affected by the calcimimetic (Table 2), confirming that the results of this study with lower doses of R568 are not confounded by hypocalcemia. The calcium levels for PCK rats were not available as samples were compromised and not eligible for measurement.

4 | DISCUSSION

In this work, we investigated, with positive outcome, whether dual targeting of V2R and CaSR could have additive effects in attenuating PKD progression in two well-established PKD animal models, the PCK rat and *Pkd1*^{RC/RC} mouse. Specifically, the main results of this

TABLE 2 Effect of lixivaptan and R568 on parameters involved in PKD development in *Pkd1*^{RC/RC} mice

Parameters	Values ± SEM	p values			
		CTR (n = 20)	LXV (n = 20)	R568 (n = 20)	LXV + R568 (n = 20)
Kidney weight/BW (%)					
CTR	1.74 ± 0.07	–	.0033*	.2835	<.0001*
LXV	1.47 ± 0.05	.0033*	–	.2866	.7348
R568	1.6 ± 0.05	.2835	.2866	–	.0309*
LXV + R568	1.39 ± 0.04	<.0001*	.7348	.0309*	–
cAMP (U/mg prot.) ^a					
CTR	427.8 ± 89.34	–	.5025	.05*	.0282*
LXV	301.6 ± 67.44	.5025	–	.6668	.3855
R568	206.6 ± 30.81	.05*	.6668	–	.8945
LXV + R568	148.5 ± 45.42	.0282*	.3855	.8945	–
Cystic volume (μl)					
CTR	22.88 ± 3.26	–	.0202*	.0099*	.0004*
LXV	13.97 ± 1.82	.0202*	–	.9914	.5874
R568	13.1 ± 1.45	.0099*	.9914	–	.771
LXV + R568	10.18 ± 1.49	.0004*	.5874	.771	–
Fibrotic volume (μl)					
CTR	5.92 ± 1.16	–	.0002*	.0002*	<.0001*
LXV	2.11 ± 0.21	.0002*	–	>.9999	.9884
R568	2.08 ± 0.24	.0002*	>.9999	–	.9918
LXV + R568	1.83 ± 0.27	<.0001*	.9884	.9918	–
Plasma calcium (mg/dl)					
CTR	7.79 ± 0.19	–	.9993	.9792	.9075
LXV	7.76 ± 0.28	.9993	–	.9507	.8452
R568	7.9 ± 0.11	.9792	.9507	–	.9918
LXV + R568	7.97 ± 0.12	.9075	.8452	.9918	–
Plasma phosphorus (mg/dl)					
CTR	7.72 ± 0.55	–	.9307	.9995	.7357
LXV	7.31 ± 0.37	.9307	–	.9607	.9747
R568	7.64 ± 0.65	.9995	.9607	–	.7981
LXV + R568	7.02 ± 0.23	.7357	.9747	.7981	–
24 h urine volume (ml)					
CTR	1.27 ± 0.14	–	<.0001*	.9976	<.0001*
LXV	3.95 ± 0.44	<.0001*	–	<.0001*	.7153
R568	1.19 ± 0.15	.9976	<.0001*	–	<.0001*
LXV + R568	3.54 ± 0.27	<.0001*	.7153	<.0001*	–
Body weight (g)					
CTR	24.5 ± 0.65	–	.8962	.9988	.9996
LXV	23.79 ± 0.74	.8962	–	.8304	.8517
R568	24.65 ± 0.81	.9988	.8304	–	>.9999
LXV + R568	24.6 ± 0.66	.9996	.8517	>.9999	–

Note: Data are expressed as means ± SEM.

^aIndirectly measured as PKA activity.

**p* < .05.

work can be summarized as follows: (i) in PCK rats, the combined treatment strongly decreased kidney weight, cyst volume and fibrosis volume by 20%, 49%, and 73%, respectively, compared to untreated animals; (ii) in *Pkd1^{RC/RC}* mice, the same parameters were reduced by 20%, 56%, and 69%, respectively; (iii) in both animal models, combination therapy appeared nominally more effective than the individual drugs used alone.

In recent years, it has become clear that disruption of vasopressin signaling has a crucial pathophysiological role in the progression of PKD and that vasopressin directly regulates cyst growth.³⁴ Accordingly, treatment with a vasopressin V2-receptor antagonist was proven to delay PKD progression in humans.^{4,5} Thus, the potentiation of the efficacy of a combination of V2R antagonist and calcimimetic compared to vaptan monotherapy can be explained by several lines of evidence showing that CaSR signaling counteracts the known vasopressin alterations^{15,35–37} in PKD. Moreover, besides collecting duct, CaSR is expressed across the entire length of the nephron, including the proximal tubule, where it is expressed apically, and the basolateral membrane of the TAL, where it has the highest expression.¹⁵ This broad expression can represent an advantage for the proposed therapeutic strategy.

The strategy to simultaneously target CaSR and V2R is strictly based on the connection between calcium signals and the onset of PKD.³ Another intriguing observation in support of this strategy is that external Ca^{2+} modulates whole cell conductance of wild type LLC-PK1 renal epithelial cells by regulating PC2-associated currents, consistent with the stimulation of CaSR.³⁸ Intracellular calcium homeostasis is impaired in ADPKD and is believed to promote cAMP accumulation and abnormal cell proliferative response to cAMP.³ However, calcium has effects beyond its impact on cAMP levels. The cellular response to cAMP is proliferative when intracellular calcium is low³⁹ and anti-proliferative when intracellular calcium is increased.⁴⁰ Therefore, there is a strong rationale in favor of targeting two GPCRs acting on each second messenger in a way that is expected to improve ADPKD dysregulations.

The PCK rat is a model orthologous to ADPKD, characterized by progressive PKD with a renal phenotype which resembles that of ADPKD.^{22,23} The *Pkd1^{RC/RC}* mouse model closely mimics human ADPKD with slowly progressive PKD.²⁴ The lixivaptan dose used in this work was established as a fully effective dose based on previous work by the authors which showed that 0.5% lixivaptan was effective in reducing cyst growth in PCK rats.¹⁰ Conversely, other previous evidence demonstrated that treatment with 0.05% and 0.1% of R568 induced profound hypocalcemia, which might have hindered a potential positive effect of the drug.²¹ Therefore, in this work we used the calcimimetic R568 at lower concentration (0.04%

for mice and 0.025% for rats) to minimize hypocalcemia. Of note, we found that plasma calcium levels in *Pkd1^{RC/RC}* mice treated with the combined treatment were not altered with respect to non-treated animals. Interestingly we wish to underline that the R-568 alone (0.04%) did not produce any effects on serum calcium levels and induced significant positive effects in reducing cAMP, cystic and fibrotic volumes, in contrast to the findings obtained by Wang et al.²¹ at higher concentration. Moreover, we report here that the 24 h urine volume was slightly but significantly reduced whereas Wang et al. reported a significant increase, supporting the positive effects of R-568 concentration used in the present contribution. On the other hand, the dual therapy did not result in an apparent improvement in plasma levels of BUN and creatinine. This is probably due to the fact that the animals were sacrificed at an age at which the worsening of BUN and creatinine levels cannot be appreciated^{24,41} and therefore the treatment on these parameters cannot be assessed.

Several in vivo studies demonstrated the pivotal role played by cAMP in PKD development, for example by promoting cyst growth through a proliferative stimulus and the secretion of fluids into the cyst lumen mediated by the cystic fibrosis transmembrane conductance regulator chloride channel.^{42–45} Both lixivaptan and R568 alone showed a clear trend in reducing cAMP levels, measured directly or by the evaluation of PKA activity, but had an additive and significant effect if used together. In addition, in both animal models, the combined treatment showed a significant increase in the expression of active AMPK, whose activity is downregulated in PKD and involved in cystogenesis.^{27,28}

Animal model differences may explain the higher efficacy of lixivaptan monotherapy in PCK rats and R568 monotherapy in *Pkd1^{RC/RC}* mice; however, the differences did not reach statistical significance. Previous work has shown that another vaptan, tolvaptan, has a dose dependent effect on kidney levels of cAMP and on PKD manifestations.⁴⁶ In one study,⁴¹ monotherapy with tolvaptan did not induce a statistical reduction of cAMP levels in *Pkd1^{RC/RC}* mice compared to control animals, whereas tolvaptan plus pasireotide combination treatment did. The rationale in this approach is that a combination of drugs targeting simultaneously the complex signaling in ADPKD, may have a potential synergism and may provide clinicians the opportunity to reduce drug doses and in turn side effects.

In this work, we show that the combined treatment with lixivaptan and R568 is indeed more effective compared to each monotherapy also in morphological disease manifestations in the kidney. In PCK rats, kidney weight reduction was statistically comparable in animals treated with lixivaptan alone or combined with R568; however, combination therapy elicited a reduction of kidney

cyst volume and fibrosis volume that reached a higher level of statistical significance compared to the effect of each monotherapy. A similar finding was observed in *Pkd1^{RC/RC}* mice with respect to kidney weight, cyst volume and fibrosis volume. Indeed, besides cystogenesis, fibrotic deposition is another major cause of renal function loss in ADPKD. Recently, V2R activation has been proven to increase renal interstitial myofibroblast population and ECM deposition in cystic epithelial cells.⁴⁷ In vitro studies in human ADPKD cystic epithelial cells showed that V2R increased fibrosis by an ERK1/2-dependent mechanism,⁴⁷ which is also regulated by CaSR signaling. Of note, in ciPTEC stably silenced for PC1, CaSR activation elicited by the calcimimetic R568 inhibited ERK1/2 pathway.¹⁷ The present study corroborates these results, demonstrating how V2R antagonism and CaSR activation may potentiate each other leading to a statistically significant fibrosis reduction only in animals treated with combination therapy, consistent with ERK1/2 activity reduction.

Despite practical considerations imposing a limit to the sample size that can be studied in an animal experiment, we are encouraged by the fact that, in both animal models, combination therapy showed a nominally greater effect for every disease-related endpoint measured in this study compared to both monotherapies and that, for every such endpoint, the treatment effect of combination therapy consistently reached a higher level of statistical significance compared to the effect of the two monotherapies. Thus, we believe that this may represent a true, important effect of combination therapy, especially when considering that the doses of lixivaptan in this study were considered fully effective doses.¹⁰

The potential additional beneficial effect of the use of calcimimetics in the treatment of PKD is strengthened by a recent elegant study demonstrating that tubular crystal deposition of calcium oxalate causes tubule dilation, activation of PKD-associated signaling pathways, and increased cystogenesis.⁴⁸ Interestingly, the authors observed that, in a cohort of ADPKD patients, lower levels of urinary excretion of citrate, an endogenous inhibitor of calcium crystal formation, were correlated with increased disease severity. These findings suggest that supplementation with citrate as a chelator of calcium to reduce renal crystal formation may slow progression in PKD. Remarkably, in the kidney, CaSR promotes citrate excretion, protecting the kidney against the risk of crystal precipitates in urine.⁴⁹ This suggests that combination therapy with R568 and lixivaptan may delay disease progression in PKD not only because CaSR counteracts vasopressin signaling (by reducing cAMP concentrations and increasing intracellular calcium), but also because it simultaneously reduces the risk of crystal deposition.

In conclusion, we demonstrated that combined treatment with lixivaptan and R568 displayed an additive

effect in reducing three of the most serious hallmarks of ADPKD, specifically renal intracellular cAMP, kidney cyst burden and kidney fibrosis. These data suggest an intriguing new clinical application for two classes of existing drugs. Vaptans and calcimimetics could be used in combination to achieve greater efficacy in the treatment of ADPKD or, in patients who cannot tolerate the aquaretic effects of a full dose vaptan treatment regimen, to reduce the vaptan dose without sacrificing therapeutic efficacy. These findings are suggestive enough to justify further investigation and possibly an exploratory clinical trial.

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DISCLOSURES

LP is an employee and owns stock in Palladio Biosciences, Inc, the sponsor of lixivaptan. R568 was supplied by Amgen Inc.

AUTHOR CONTRIBUTIONS

Annarita Di Mise, Vicente E. Torres and Giovanna Valenti designed research and supervised the project; Annarita Di Mise, Xiaofang Wang and Hong Ye performed research and analyzed data; Annarita Di Mise, Vicente E. Torres and Giovanna Valenti designed and interpreted experiments; Annarita Di Mise, Vicente E. Torres and Giovanna Valenti wrote and revised the paper; Lorenzo Pellegrini revised the paper; all authors commented on the manuscript.

ORCID

Annarita Di Mise  <https://orcid.org/0000-0002-8132-4917>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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